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Production and secretion of plasminogen in cultured rat brain microglia

Kazuyuki Nakajima, Naoko Tsuzaki, Koichi Nagata, Nagisa Takemoto and Shinichi Kohsaka

Department of Neurochemistry, National Institute of Neuroscience, Kodaira, Tokyo 187, Japan

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The production and secretion of plasminogen in cultured rat brain microglia was investigated. Urokinase-dependent caseinolytic activity was detected by zymography in microglial conditioned medium with a molecular weight of about 90 kDa. The 90-kDa protein was also detected by Western blotting with anti-rat plasminogen antiserum in the non-reducing condition. Immunoprecipitation with plasminogen antiserum following [^{35}S]methionine labelling revealed that the plasminogen detected in microglial conditioned medium is synthesized in microglia. The amount of plasminogen in the conditioned medium was increased by stimulation with lipopolysaccharide. These results show that cultured microglia produce plasminogen and secrete it into the culture medium.

Protease; Plasminogen; Plasminogen activator; Culture; Microglia

1. INTRODUCTION

Accumulating lines of evidence suggest that the plasminogen–plasminogen activator (PA) system plays important roles in many biological reactions, such as fibrinolysis [1], extracellular proteolysis, cell migration, cell invasion, tissue remodeling [2,3] and the processing of proenzymes [4,5], prohormone [6], progrowth factor [7], and procytokine [8]. In the central nervous system (CNS), the system is also thought to be associated with the processes of neurite outgrowth [9–11], cell migration [12], and astroglial proliferation [13].

Microglia, one type of glial cells in the CNS, are detected in a scattered fashion as ramified microglia in the brain, and they are activated in various pathological states and become ameboid microglia, which are believed to scavenge cell debris in an injured site [14]. In recent years, much attentin has been paid to the involvement of microglia in causing neurological disorders such as AIDS encephalitis [15], Alzheimer's disease [16] and multiple sclerosis [17]. In vitro studies are necessary to determine the precise roles of microglia in normal development and the pathological state in the CNS. Isolated and cultured microglia show morphological similarity to ameboid microglia [18–23], and are reported to produce cytokines [24–26] and growth factors [27], and to have antigen presentation [20,21,28] and phagocytotic ability [23,29]. These properties seem to reflect the in vivo functions of microglia.

Our recent studies indicated that microglia isolated from rat brain primary culture secrete a PA which seems to be related to such biological events as extracellular proteolysis, morphological changes and cell migration [30]. However, the production of plasminogen, the substrate of PA, in microglia remained unclear. In the present study we found that plasminogen is produced in cultured microglia and is secreted extracellularly.

Correspondence address: S. Kohsaka, Department of Neurochemistry, National Institute of Neuroscience, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187, Japan. Fax: (81) (423) 46 1751.

2. MATERIALS AND METHODS

2.1. *Preparation of microglia and their conditioned medium*

Isolation of microglia from a primary culture of rat brain, and the preparation of microglial conditioned medium (Mic-CM) were described previously [23,31]. In brief, isolated and cultured microglia ($3–5 \times 10^6$ cells/75 cm^2) were rinsed three times with serum-free DMEM and cultured in the same medium for 0–48 h. The harvested medium was concentrated about 30-fold by Amicon ultrafiltration (YM 5 membrane), and used either immediately or after being freeze-dried.

2.2. *Casein-urokinase-zymography*

In order to detect plasminogen and estimate its molecular weight, casein-urokinase-zymography was performed according to a modification of the method described elsewhere [32,33]. Mic-CM and plasminogen purified from rat plasma were subjected to SDS-PAGE under non-reducing conditions. The polyacrylamide gels were washed for 40 min with 2.5% Triton X-100 in 50 mM Tris-HCl (pH 7.5) buffer and overlaid on a 1% agarose gel containing 100 U of human urokinase and 20 mg of casein per ml. In the casein-plasminogen-zymography used to detect PA, human urokinase was replaced by 100 μg of human plasminogen per ml. The layered gels were incubated for 1–6 h at 37°C in a humidified chamber. The agarose gel was stained with 1% Amido black in 70% methanol and 10% acetic acid.

2.3. *Preparation of antiserum against rat plasminogen*

Rat plasminogen was purified from the plasma of adult rats. The plasma was directly applied onto a lysine-Sepharose column (2 × 10 cm) [34] equilibrated with 50 mM potassium phosphate buffer (pH 7.4) (KPB). After being washed with KPB and with KPB containing 0.5 M NaCl, plasminogen was eluted with 0.2 M 6-amino caproic acid. Plasminogen fractions were precipitated with 80% saturated ammo-

nium sulfate, and further purified by gel filtration using Sephadex G-150.

Polyclonal antibody to the purified rat plasminogen was raised in rabbits. New Zealand White rabbits were immunized by injection of 1 mg of the plasminogen emulsified in Freund's complete adjuvant, and boosted three times at 2-week intervals with approximately 200 μg of plasminogen in incomplete Freund's adjuvant.

2.4. *Western blotting*

Proteins were electrophoresed by SDS-PAGE [35], and transferred to Immobilon-P (Millipore) with a semi-dry transblotting apparatus as described previously [31]. The antibody binding was detected with 0.02% 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2.

2.5. *Immunoprecipitation*

Microglia (5×10^6 cells per 75 cm^2) were labelled with 15 MBq of [^{35}S]methionine (>37 TBq/mmol, Amersham) in 5 ml of methionine-free DMEM for 24 h. The conditioned medium was collected and concentrated as described above, and freeze-dried plasminogen in the Mic-CM was immunoprecipitated as described previously [31]. The immune complexes were subjected to SDS-PAGE after being heated in Laemmli's sample solution at 90°C for 2 min [36]. [^{35}S]Methionine-labelled bands were detected by autoradiography.

3. RESULTS

3.1. *Detection of plasminogen by zymography*

Mic-CM was examined for plasminogen by zymography. As shown in Fig. 1 (lane 3), Mic-CM formed a lytic band in casein-urokinase zymography at the position of about 90 kDa, which corresponded to plasminogen purified from rat plasma (lane 1). No lytic band was observed when urokinase was omitted from the agarose gel (lane 4), while in casein-plasminogen-zymography, the same conditioned medium formed a lytic band at about 47 kDa which is possibly PA (lane 2) as we reported previously [30].

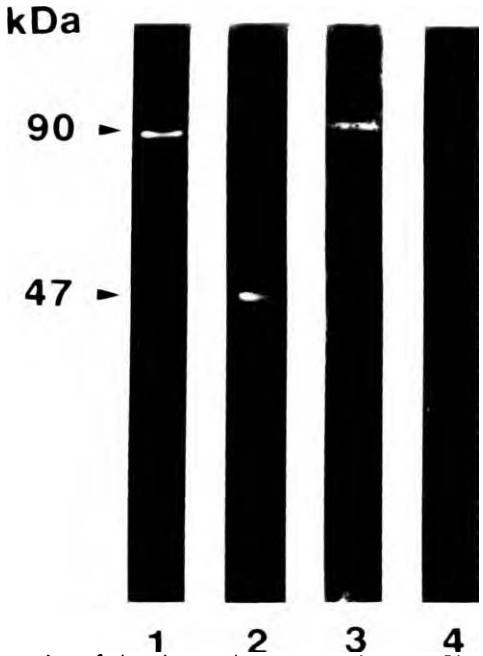

Fig. 1. Detection of plasminogen by zymography. Mic-CM (70 μg of protein) was examined by zymography for the presence (lane 3) or absence (lane 4) of urokinase. In lane 1, plasminogen (20 ng) purified from plasma is shown. For lane 2, plasminogen activator was analyzed by using the same Mic-CM (3 μg protein) as in lane 3.

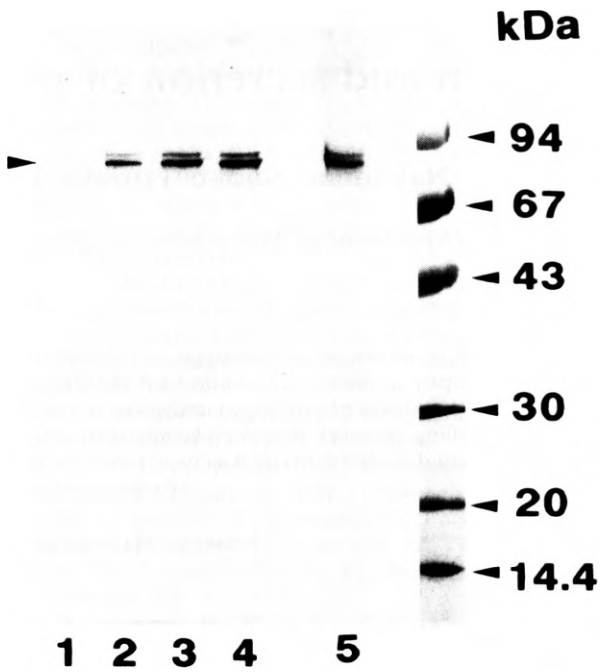

Fig. 2. Immuno-blot testing of Mic-CM for plasminogen. Microglia (4×10^5 cells) were seeded in the wells of a 6-well plate and maintained with serum-free DMEM for various times. Mic-CM, recovered at 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), and 48 h (lane 4) were subjected to SDS-PAGE under non-reducing conditions and immunoblotted. In lane 5, 10 ng of rat plasminogen was used. Molecular weight marker proteins phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) are shown on the right side.

3.2. *Immuno-blot testing of Mic-CM for plasminogen*

The existence of plasminogen and the changes in the amount of plasminogen in Mic-CM with culture time were investigated by Western blotting. As shown in Fig. 2, the intensity of immunoreactivity significantly increased with culture time (0–48 h), suggesting that microglia secrete plasminogen in a time-dependent manner. The amounts of plasminogen secreted were roughly estimated to be 5 ng/4 × 10^5 cells/2 days, using purified plasminogen as a calibration control.

3.3. *De novo synthesis of plasminogen*

To demonstrate that the detected plasminogen was derived from microglia, [^{35}S]methionine was added to the culture medium and Mic-CM collected 24 h later was subjected to immunoprecipitation. As shown in Fig. 3 (lane 1), proteins of about 90 kDa which were labelled with [^{35}S]methionine were precipitated by rat plasminogen antiserum, although several [^{35}S]methionine-labelled bands were detected in the Mic-CM (lane 3). Furthermore, when the same Mic-CM was subjected to casein-urokinase zymography, plasminogen was

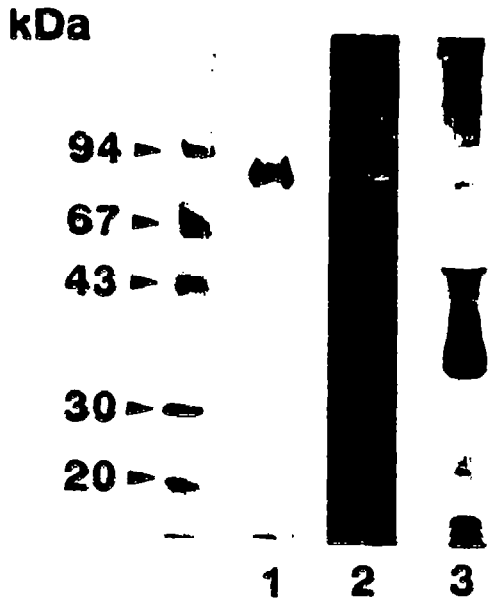

Fig. 3. De novo synthesis of plasminogen. Molecular marker proteins on the left side are the same as in Fig. 2. [^{35}S]Methionine-labelled proteins in Mic-CM were divided into two parts. One part was immunoprecipitated with plasminogen antiserum as described in section 2, and the precipitated ^{35}S-labelled proteins were analyzed by SDS-PAGE followed by autoradiography (lane 1). The other part was subjected to SDS-PAGE in a non-reducing condition and zymographed (lane 2). The polyacrylamide gel used in zymography was autoradiographed (lane 3).

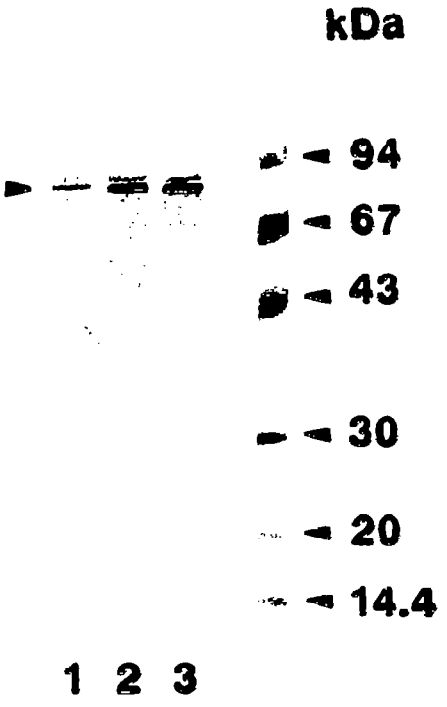

Fig. 4. LPS stimulation of plasminogen secretion. Microglia (1.5×10^5) were seeded in the wells of a 12-well plate. After the medium was replaced with serum-free DMEM, LPS was added at concentrations of 0–10 μg/ml and the cultures were maintained for 36 h. The recovered medium, which was either not stimulated (lane 1) or stimulated with 2 μg of LPS/ml (lane 2) or 10 μg of LPS/ml (lane 3) was analyzed by Western blotting as in Fig. 2.

clearly detected at about 90 kDa (lane 2). These results indicate that the plasminogen detected in Mic-CM is synthesized in microglia.

3.4. *Stimulation of plasminogen secretion by LPS*

The effect of lipopolysaccharide (LPS) stimulation on the amount of plasminogen in Mic-CM was investigated by Western blotting. Although the number of cells was not affected (data not shown), the amount of plasminogen in Mic-CM was found to be increased about 2- to 3-fold by the addition of LPS (2–10 μg per ml) (Fig. 4). These results suggest that the amount of secretory plasminogen from microglia is regulated by various effectors.

4. DISCUSSION

The plasminogen–PA system is generally accepted as being connected mainly with fibrinolysis, and with other biological events such as extracellular proteolysis and cell migration. Blood plasminogen is thought to be derived from liver cells. It is reported that liver [37] and hepatocytes [38] do in fact produce plasminogen. The plasminogen–PA system is also thought to be associated with cell proliferation [13], cell migration [12], and neurite extension [9–11] in the CNS. Although it has been suggested that plasminogen is produced in neuronal cells, its production in microglial cells remained unclear. In the present study, we have demonstrated that plasminogen is produced in and secreted from microglia, one type of glial cells in the CNS. By zymography and Western blotting, microglia-derived plasminogen was detected as a doublet around 90 kDa, like rat plasma-derived plasminogen. These two forms were thought to depend on the difference in glycosylation as reported for human plasminogen [39], in which two major glycoforms have an identical amino-acid sequence. There was a possibility that plasminogen detected in Mic-CM came from the fetal calf serum used for culture medium of the primary culture. However, we excluded that possibility by [^{35}S]methionine labelling and immunoprecipitation experiments.

What is the physiological meaning of the microglia-derived plasminogen? The most plausible speculation is the one in relation to the degradation of macromolecules like those in cell or tissue debris, because microglia are known to be activated as scavenger cells in response to brain injury or pathological stimuli.

LPS is a strong stimulus for microglia, and leads to an increase in the secretion of IL-1 [22], TNF [26] and NGF [27] by microglia. As shown in the present study, LPS also stimulates the secretion of plasminogen from microglia. Therefore, it is likely that the secretion of plasminogen by microglia is regulated in response to

various effectors, which may originate in the surrounding cells. Furthermore, a number of studies have shown that plasmin can process and activate procollagenase [4], pro uPA [5], proinsulin [6], pro IL-8 [8] and latent TGF_β [7]. Taking into account these studies, it is also possible to speculate that the plasminogen–PA system secreted from microglia plays important roles not only in migration and extracellular proteolysis but also in the regulation of growth factors, cytokines and hormones activities in the extracellular spaces.

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REFERENCES

[1] Collen, D. (1980) Thromb. Haemostasis 43, 77–89.
[2] Reich, E. (1973) Fed. Proc. 32, 2174–2175.
[3] Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res. 44, 139–266.
[4] Liotta, L.A., Goldfarb, R.H., Brundage, R., Siegal, G.P., Terranova, V. and Garbisa, S. (1981) Cancer Res. 41, 4629–4636.
[5] Blasi, F., Vassalli, J.-D. and Dano, K. (1987) J. Cell Biol. 104, 801–804.
[6] Virji, M.A.G., Vassalli, J.-D., Estensen, R.D. and Reich, E. (1980) Proc. Natl. Acad. Sci. USA 77, 875–879.
[7] Rifkin, D.B., Moscatelli, D., Bizik, J., Quarto, N., Blei, F., Dennis, P., Flaumenhaft, R. and Mignatti, P. (1990) Cell Differ. Dev. 32, 313–318.
[8] Nakagawa, H., Hatakeyama, S., Ikesue, A. and Miyai, H. (1991) FEBS Lett. 282, 412–414.
[9] Kryostosek, A. and Seeds, N.W. (1981) Science 213, 1532–1534.
[10] Pittman, R.N. (1985) Dev. Biol. 110, 91–101.
[11] Pittman, R.N., Ivins, J.K. and Buettner, H.M. (1989) J. Neurosci. 9, 4269–4286.
[12] Kalderon, N. (1979) Proc. Natl. Acad. Sci. USA 76, 5992–5996.
[13] Kalderon, N. (1982) J. Neurosci. Res. 8, 509–519.
[14] Rio-Hortega, P. (1932) in: Cytology and Cellular Pathology of the Nervous System (W. Penfield, Ed.) Microglia, vol. 2, pp. 483–534, Hocker, NY.
[15] Dickson, D.W., Mattiace, L.A., Kure, K., Hutchins, K., Lyman, W.D. and Brosnan, F. (1991) Lab. Invest. 64, 135–156.
[16] Wisniewski, H.M., Wegiel, J., Wang, K.C., Kujawa, M. and Lach, B. (1989) Can. J. Neurol. Sci. 16, 535–542.
[17] Woodroofe, M.N., Bellamy, A.S., Feldmann, M., Davison, A.N. and Cuzner, M.L. (1986) J. Neurol. Sci. 74, 135–152.
[18] Ling, E.A., Tseng, C.Y., Yoon, F.C.T. and Wong, W.C. (1983) J. Anat. 137, 223–233.
[19] Giulian, G. and Baker, T.J. (1986) J. Neurosci. 6, 2163–2178.
[20] Suzumura, A., Mezitis, S.G.E., Gonatas, N.K. and Silberberg, D.H. (1987) J. Neuroimmunol. 15, 263–278.
[21] Frei, K., Siepl, C., Groscurth, P., Bodmer, S., Schwerdel, C. and Fontana, A. (1987) Eur. J. Immunol. 17, 1271–1278.
[22] Gebicke-Haerter, P.J., Bauer, J., Schobert, A. and Northoff, H. (1989) J. Neurosci. 9, 183–194.
[23] Nakajima, K., Hamanoue, M., Shimojo, M., Takei, N. and Kohsaka, S. (1989) Biomed. Res. 10(S3), 411–423.
[24] Giulian, D., Baker, T.J., Shin, L.-C.N. and Lachman, L.B. (1986) J. Exp. Med. 164, 594–604.
[25] Frei, K., Malipiero, U.V., Leist, T.P., Zinkernagel, R.M., Schwab, M.E. and Fontana, A. (1989) Eur. J. Immunol. 19, 689–694.
[26] Sawada, M., Kondo, N., Suzumura, A. and Marunouchi, T. (1989) Brain Res. 491, 394–397.
[27] Mallat, M., Houlgatte, R., Brachet, P. and Prochiantz, A. (1989) Dev. Biol. 133, 309–311.
[28] Hickey, W.F. and Kimura, H. (1988) Science 239, 290–292.
[29] Rieske, E., Grueber, M.B., Tetzlaff, W., Czlonkowska, A., Streit, W.J. and Kreutzberg, G.W. (1989) Brain Res. 492, 1–14.
[30] Nakajima, K., Tsuzaki, N., Shimojo, M., Hamanoue, M. and Kohsaka, S. (1992) Brain Res. 577, 285–292.
[31] Nakajima, K., Shimojo, M., Hamanoue, M., Ishiura, S., Sugita, H. and Kohsaka, S. (1992) J. Neurochem. 58, 1401–1408.
[32] Granelli-Pipeno and Reich (1978) J. Exp. Med. 148, 223–234.
[33] Vassalli, J.-D., Dayer, J.-M., Wohlwend, A. and Belin, D. (1984) J. Exp. Med. 159, 1653–1668.
[34] Deutsch, D.G. and Mertz, E.T. (1970) 170, 1095–1096.
[35] Laemmli, U.K. (1970) Nature 227, 680–685.
[36] Gebicke-Haerter, P.J., Bauer, J., Bernner, A. and Gerok, W. (1987) J. Neurochem. 49, 1139–1145.
[37] Tomlinson, J.E., McLean, J.W. and Lawn, R.M. (1989) J. Biol. Chem. 264, 5957–5965.
[38] Bohmfald, J.F. and Fuller, G.M. (1980) Science 209, 408–410.
[39] Castellino, F.J. (1983) Bioscience 33, 647–650.